

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C07K 7/10, C12N 15/15 A61K 37/64	A1	(11) International Publication Number: WO 93/14121 (43) International Publication Date: 22 July 1993 (22.07.93)
(21) International Application Number: PCT/DK93/00004 (22) International Filing Date: 7 January 1993 (07.01.93) (30) Priority data: PCT/DK92/00001 7 January 1992 (07.01.92) WO (34) Countries for which the regional or international application was filed: AT et al. (71) Applicant (for all designated States except US): NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsvaerd (DK). (72) Inventors; and (75) Inventors/Applicants (for US only) : NORRIS, Fanny [DK/DK]; NORRIS, Kjeld [DK/DK]; Ahlmanns Allé 34, DK-2900 Hellerup (DK). BJØRN, Søren, Erik [DK/DK]; Marie Grubbes Allé 47, DK-2800 Lyngby (DK). PETERSEN, Lars, Christian [DK/DK]; Havevej 4, DK-2970 Hørsholm (DK). OLSEN, Ole, Hvilsted [DK/DK]; Bækkeskovvej 38, DK-2700 Brønshøj (DK).		(74) Common Representative: NOVO NORDISK A/S; Patent Department, Novo Allé, DK-2880 Bagsvaerd (DK). (81) Designated States: AU, CA, CZ, FI, HU, JP, KR, NO, NZ, PL, RU, SK, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: A HUMAN KUNITZ-TYPE PROTEASE INHIBITOR VARIANT (57) Abstract <p>Variant of human Kunitz-type protease inhibitor domain II of tissue factor pathway inhibitor (TFPI), the variant comprising the following amino acid sequence X¹ Asp Phe Cys Phe Leu Glu Glu Asp X² Gly X³ Cys X⁴ X⁵ X⁶ X⁷ X⁸ X⁹ Tyr Phe Tyr Asn Asn Gln Thr Lys Gln Cys Glu Arg Phe X¹⁰ Tyr Gly Gly Cys X¹¹ X¹² X¹³ Met Asn Asn Phe X¹⁴ Thr Leu Glu Glu Cys Lys Asn Ile Cys Glu Asp X¹⁵ (SEQ ID No. 1), wherein X¹ represents H or 1-5 naturally occurring amino acid residues except Cys, X²-X¹⁴ each independently represents a naturally occurring amino acid residue, and X¹⁵ represents OH or 1-5 naturally occurring amino acid residues except Cys, with the proviso that at least one of the amino acid residues X¹-X¹⁵ is different from the corresponding amino acid residue of the native sequence.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SK	Slovak Republic
CI	Côte d'Ivoire	LI	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	MC	Monaco	TG	Togo
DE	Germany	MG	Madagascar	UA	Ukraine
DK	Denmark	ML	Mali	US	United States of America
ES	Spain	MN	Mongolia	VN	Viet Nam
FI	Finland				

A HUMAN KUNITZ-TYPE PROTEASE INHIBITOR VARIANT

FIELD OF INVENTION

5

The present invention relates to a variant of a human Kunitz-type protease inhibitor domain, DNA encoding the variant, a method of producing the variant and a pharmaceutical composition containing the variant.

10

BACKGROUND OF THE INVENTION

Polymorphonuclear leukocytes (neutrophils or PMNs) and mononuclear phagocytes (monocytes) play an important part in tissue injury, infection, acute and chronic inflammation and wound healing. The cells migrate from the blood to the site of inflammation and, following appropriate stimulation, they release oxidant compounds (O_2^* , O_2^- , H_2O_2 and $HOCl$) as well as granules containing a variety of proteolytic enzymes. The secretory granules contain, i.a., alkaline phosphatase, metalloproteinases such as gelatinase and collagenase and serine proteases such as neutrophil elastase, cathepsin G and proteinase 3.

Latent metalloproteinases are released together with tissue inhibitor of metalloproteinase (TIMP). The activation mechanism has not been fully elucidated, but it is likely that oxidation of thiol groups and/or proteolysis play a part in the process. Also, free metalloproteinase activity is dependent on inactivation of TIMP.

In the azurophil granules of the leukocytes, the serine proteases neutrophil elastase, cathepsin G and proteinase-3 are packed as active enzymes complexed with glucosaminoglycans. These complexes are inactive but dissociate on secretion to release the active enzymes. To neutralise the protease activity, large amounts of the inhibitors α_1 -proteinase inhibitor (α_1 -PI)

and α_1 -chymotrypsin inhibitor (α_1 -ChI) are found in plasma. However, the PMNs are able to inactivate the inhibitors locally. Thus, α_1 -PI which is the most important inhibitor of neutrophil elastase is sensitive to oxidation at the reactive centre (Met-
5 358) by oxygen metabolites produced by triggered PMNs. This reduces the affinity of α_1 -PI for neutrophil elastase by approximately 2000 times.

After local neutralisation of α_1 -PI, the elastase is able to
10 degrade a number of inhibitors of other proteolytic enzymes. Elastase cleaves α_1 -ChI and thereby promotes cathepsin G activity. It also cleaves TIMP, resulting in tissue degradation by metalloproteinases. Furthermore, elastase cleaves antithrombin III and heparin cofactor II, and tissue factor
15 pathway inhibitor (TFPI) which probably promotes clot formation. On the other hand, the ability of neutrophil elastase to degrade coagulation factors is assumed to have the opposite effect so that the total effect of elastase is unclear. The effect of neutrophil elastase on fibrinolysis is less ambiguous.
20 Fibrinolytic activity increases when the elastase cleaves the plasminogen activator inhibitor and the α_2 plasmin inhibitor. Besides, both of these inhibitors are oxidated and inactivated by O_2 metabolites.

25 PMNs contain large quantities of serine proteases, and about 200 mg of each of the leukocyte proteases are released daily to deal with invasive agents in the body. Acute inflammation leads to a many-fold increase in the amount of enzyme released. Under normal conditions, proteolysis is kept at an acceptably low
30 level by large amounts of the inhibitors α_1 -PI, α_1 -ChI and α_2 macroglobulin. There is some indication, however, that a number of chronic diseases is caused by pathological proteolysis due to overstimulation of the PMNs, for instance caused by autoimmune response, chronic infection, tobacco smoke or other irritants,
35 etc.

Aprotinin (bovine pancreatic trypsin inhibitor) is known to

- inhibit various serine proteases, including trypsin, chymotrypsin, plasmin and kallikrein, and is used therapeutically in the treatment of acute pancreatitis, various states of shock syndrome, hyperfibrinolytic haemorrhage and myocardial infarction (cf., for instance, J.E. Trapnell et al, 5 Brit. J. Surg. 61, 1974, p. 177; J. McMichan et al., Circulatory shock 9, 1982, p. 107; L.M. Auer et al., Acta Neurochir. 49, 1979, p. 207; G. Sher, Am. J. Obstet. Gynecol. 129, 1977, p. 164; and B. Schneider, Artzheim.-Forsch. 26, 1976, p. 1606).
- 10 Administration of aprotinin in high doses significantly reduces blood loss in connection with cardiac surgery, including cardiopulmonary bypass operations (cf., for instance, B.P. Bidstrup et al., J. Thorac. Cardiovasc. Surg. 97, 1989, pp. 364-372; W. van Oeveren et al., Ann. Thorac. Surg. 44, 1987, pp. 15 640-645). It has previously been demonstrated (cf. H.R. Wenzel and H. Tschesche, Angew. Chem. Internat. Ed. 20, 1981, p. 295) that certain aprotinin analogues, e.g. aprotinin(1-58, Val15) exhibits a relatively high selectivity for granulocyte elastase and an inhibitory effect on collagenase, aprotinin (1-58, Ala15) 20 has a weak effect on elastase, while aprotinin (3-58, Arg15, Ala17, Ser42) exhibits an excellent plasma kallikrein inhibitory effect (cf. WO 89/10374).

However, when administered in vivo, aprotinin has been found to 25 have a nephrotoxic effect in rats, rabbits and dogs after repeated injections of relatively high doses of aprotinin (Bayer, Trasylol, Inhibitor of proteinase; E. Glaser et al. in "Verhandlungen der Deutschen Gesellschaft für Innere Medizin, 78. Kongress", Bergmann, München, 1972, pp. 1612-1614). The 30 nephrotoxicity (i.a. appearing in the form of lesions) observed for aprotinin might be ascribed to the accumulation of aprotinin in the proximal tubulus cells of the kidneys as a result of the high positive net charge of aprotinin which causes it to be bound to the negatively charged surfaces of the tubuli.. This 35 nephrotoxicity makes aprotinin less suitable for clinical purposes, in particular those requiring administration of large doses of the inhibitor (such as cardiopulmonary bypass

operations). Besides, aprotinin is a bovine protein which may therefore contain one or more epitopes which may give rise to an undesirable immune response on administration of aprotinin to humans.

5

It is therefore an object of the present invention to identify human protease inhibitors of the same type as aprotinin (i.e. Kunitz-type inhibitors) with a similar inhibitor profile or modified to exhibit a desired inhibitor profile.

10

SUMMARY OF THE INVENTION

The present invention relates to a variant of human Kunitz-type protease inhibitor domain II of tissue factor pathway inhibitor (TFPI), the variant comprising the following amino acid sequence

X¹ Asp Phe Cys Phe Leu Glu Glu Asp X² Gly X³ Cys X⁴ X⁵ X⁶ X⁷ X⁸ X⁹
Tyr Phe Tyr Asn Asn Gln Thr Lys Gln Cys Glu Arg Phe X¹⁰ Tyr Gly
Gly Cys X¹¹ X¹² X¹³ Met Asn Asn Phe X¹⁴ Thr Leu Glu Glu Cys Lys Asn
20 Ile Cys Glu Asp X¹⁵ (SEQ ID No. 1)

wherein X¹ represents H or 1-5 naturally occurring amino acid residues except Cys, X²-X¹⁴ each independently represents a naturally occurring amino acid residue except Cys, and X¹⁵
25 represents OH or 1-5 naturally occurring amino acid residues except Cys, with the proviso that at least one of the amino acid residues X¹-X¹⁵ is different from the corresponding amino acid residue of the native sequence.

30 In the present context, the term "naturally occurring amino acid residue" is intended to indicate any one of the 20 commonly occurring amino acids, i.e. Ala, Val, Leu, Ile Pro, Phe, Trp, Met, Gly, Ser, Thr, Cys, Tyr, Asn, Gln, Asp, Glu, Lys, Arg and His.

35

TFPI, also known as extrinsic pathway inhibitor (EPI) or lipoprotein associated coagulation inhibitor (LACI), has been

isolated by Broze et al. (Proc. Natl. Acad. Sci. USA 84, 1987, pp. 1886-1890 and EP 300 988) and the gene coding for the protein has been cloned, cf. EP 318 451. Analysis of the secondary structure of the protein has shown that the protein
5 has three Kunitz-type inhibitor domains, from amino acid 22 to amino acid 79 (I), from amino acid 93 to amino acid 150 (II) and from amino acid 185 to amino acid 242 (III). Kunitz-type domain I of TFPI has been shown to bind TF/FVIIa, while Kunitz-type domain II has been shown to bind to FXa (Girard et al., Nature
10 338, 1989, pp. 518-520).

By substituting one or more amino acids in one or more of the positions indicated above, it may be possible to change the inhibitor profile of TFPI Kunitz-type domain II so that it
15 preferentially inhibits neutrophil elastase, cathepsin G and/or proteinase-3. Furthermore, it may be possible to construct variants which specifically inhibit enzymes involved in coagulation or fibrinolysis (e.g. plasmin or plasma kallikrein) or the complement cascade.

20

One advantage of TFPI Kunitz-type domain II is that it has a negative net charge as opposed to aprotinin which, as indicated above, has a strongly positive net charge. It is therefore possible to construct variants of the invention with a lower
25 positive net charge than aprotinin, thereby reducing the risk of kidney damage on administration of large doses of the variants. Another advantage is that, contrary to aprotinin, it is a human protein (fragment) so that undesired immunological reactions on administration to humans are significantly reduced.

30

DETAILED DISCLOSURE OF THE INVENTION

Examples of preferred variants of Kunitz-type domain II of TFPI are variants wherein X^1 is Lys-Pro; or wherein X^2 is an amino
35 acid residue selected from the group consisting of Ala, Arg, Thr, Asp, Pro, Glu, Lys, Gln, Ser, Ile and Val, in particular wherein X^2 is Thr or Pro; or wherein X^3 is an amino acid residue

selected from the group consisting of Pro, Thr, Leu, Arg, Val and Ile, in particular wherein X^3 is Pro or Ile; or wherein X^4 is an amino acid residue selected from the group consisting of Lys, Arg, Val, Thr, Ile, Leu, Phe, Gly, Ser, Met, Trp, Tyr, Gln, Asn and Ala, in particular wherein X^4 is Lys, Val, Leu, Ile, Thr, Met, Gln or Arg; or wherein X^5 is an amino acid residue selected from the group consisting of Ala, Gly, Thr, Arg, Phe, Gln and Asp, in particular wherein X^5 is Ala, Thr, Asp or Gly; or wherein X^6 is an amino acid residue selected from the group consisting of Arg, Ala, Lys, Leu, Gly, His, Ser, Asp, Gln, Glu, Val, Thr, Tyr, Phe, Asn, Ile and Met, in particular wherein X^6 is Arg, Phe, Ala, Leu or Tyr; or wherein X^7 is an amino acid residue selected from the group consisting of Ile, Met, Gln, Glu, Thr, Leu, Val and Phe, in particular wherein X^7 is Ile; or wherein X^8 is an amino acid residue selected from the group consisting of Ile, Thr, Leu, Asn, Lys, Ser, Gln, Glu, Arg, Pro and Phe, in particular wherein X^8 is Ile or Thr; or wherein X^9 is an amino acid residue selected from the group consisting of Arg, Ser, Ala, Gln, Lys and Leu, in particular wherein X^9 is Arg; or wherein X^{10} is an amino acid residue selected from the group consisting of Gln, Pro, Phe, Ile, Lys, Trp, Ala, Thr, Leu, Ser, Tyr, His, Asp, Met, Arg and Val, in particular wherein X^{10} is Val or Lys; or wherein X^{11} is an amino acid residue selected from the group consisting of Gly, Met, Gln, Glu, Leu, Arg, Lys, Pro and Asn, in particular wherein X^{11} is Arg or Leu; or wherein X^{12} is Ala or Gly; or wherein X^{13} is an amino acid residue selected from the group consisting of Lys, Asn and Asp, in particular wherein X^{13} is Lys or Asn; or wherein X^{14} is an amino acid residue selected from the group consisting of Val, Tyr, Asp, Glu, Thr, Gly, Leu, Ser, Ile, Gln, His, Asn, Pro, Phe, Met, Ala, Arg, Trp and Lys, in particular wherein X^{14} is Lys or Glu; or wherein X^{15} is Gly. In a preferred embodiment, X^1 is Lys-Pro and X^{15} is Gly, while X^2 - X^{14} are as defined above.

35 Variants of TFPI Kunitz-type domain II of the invention should preferably not contain a Met residue in the protease binding region (i.e. the amino acid residues represented by X^3 - X^{14}). By

analogy to α 1-PI described above, a Met residue in any one of these positions would make the inhibitor sensitive to oxidative inactivation by oxygen metabolites produced by PMNs, and conversely, lack of a Met residue in these positions should
 5 render the inhibitor more stable in the presence of such oxygen metabolites.

It may be desired to change the way in which the TFPI Kunitz-type domain II variant is glycosylated when produced by a host
 10 cell. Thus, in one embodiment, the variant of the invention may have the following amino acid sequence

X¹ Asp Phe Cys Phe Leu Glu Glu Asp X² Gly X³ Cys X⁴ X⁵ X⁶ X⁷ X⁸ X⁹
 Tyr Phe Tyr Asn X¹⁶ Gln X¹⁷ Lys Gln Cys Glu Arg Phe X¹⁰ Tyr Gly Gly
 15 Cys X¹¹ X¹² X¹³ Met Asn Asn Phe X¹⁴ Thr Leu Glu Glu Cys Lys Asn Ile
 Cys Glu Asp X¹⁵ (SEQ ID No. 2)

wherein X¹-X¹⁵ are as indicated in claim 1, X¹⁶ is an amino acid residue selected from the group consisting of Gln, Gly, Ala, Ser, Val and Phe, in particular Gln or Ala, and X¹⁷ is an amino
 20 acid residue selected from the group consisting of Thr or Ala.

Currently preferred variants of the invention are those in which one or more the amino acid residues located at the protease-binding site of the Kunitz domain (i.e. one or more of X³-X¹⁴
 25 corresponding to positions 13, 15, 16, 17, 18, 19, 20, 34, 39, 40, 41 and 46 of aprotinin) are substituted to the amino acids present in the same position(s) of native aprotinin. Examples of such variants are

30

Lys Pro Asp Phe Cys Phe Leu Glu Glu Asp Pro Gly Ile Cys Lys Ala
 Arg Ile Thr Arg Tyr Phe Tyr Asn Asn Gln Thr Lys Gln Cys Glu Arg
 Phe Lys Tyr Gly Gly Cys Leu Gly Asn Met Asn Asn Phe Glu Thr Leu
 Glu Glu Cys Lys Asn Ile Cys Glu Asp Gly (SEQ ID No. 3);

35

Lys Pro Asp Phe Cys Phe Leu Glu Glu Asp Pro Gly Ile Cys Lys Ala
 Arg Ile Thr Arg Tyr Phe Tyr Asn Asn Gln Thr Lys Gln Cys Glu Arg

Phe Lys Tyr Gly Gly Cys Arg Gly Asn Met Asn Asn Phe Glu Thr Leu
Glu Glu Cys Lys Asn Ile Cys Glu Asp Gly (SEQ ID No. 4);

5 Lys Pro Asp Phe Cys Phe Leu Glu Glu Asp Pro Gly Ile Cys Lys Ala
Arg Ile Thr Arg Tyr Phe Tyr Asn Asn Gln Thr Lys Gln Cys Glu Arg
Phe Lys Tyr Gly Gly Cys Arg Gly Asn Met Asn Asn Phe Lys Thr Leu
Glu Glu Cys Lys Asn Ile Cys Glu Asp Gly (SEQ ID No. 5); or

10 Lys Pro Asp Phe Cys Phe Leu Glu Glu Asp Pro Gly Pro Cys Lys Ala
Arg Ile Ile Arg Tyr Phe Tyr Asn Asn Gln Thr Lys Gln Cys Glu Arg
Phe Val Tyr Gly Gly Cys Arg Ala Lys Met Asn Asn Phe Lys Thr Leu
Glu Glu Cys Lys Asn Ile Cys Glu Asp Gly (SEQ ID No. 6).

15 In another aspect, the invention relates to a DNA construct
encoding a human Kunitz-type inhibitor domain variant according
to the invention. The DNA construct of the invention may be
prepared synthetically by established standard methods, e.g. the
phosphoamidite method described by S.L. Beaucage and M.H.
Caruthers, Tetrahedron Letters 22, 1981, pp. 1859-1869, or the
20 method described by Matthes et al., EMBO Journal 3, 1984, pp.
801-805. According to the phosphoamidite method,
oligonucleotides are synthesized, e.g. in an automatic DNA
synthesizer, purified, annealed, ligated and cloned in suitable
vectors.

25 Alternatively, it is possible to use genomic or cDNA coding for
TFPI Kunitz-type domain II (e.g. obtained by screening a genomic
or cDNA library for DNA coding for TFPI using synthetic
oligonucleotide probes and isolating the DNA sequence coding for
30 domain II therefrom). The DNA sequence is modified at one or
more sites corresponding to the site(s) at which it is desired
to introduce amino acid substitutions, e.g. by site-directed
mutagenesis using synthetic oligonucleotides encoding the
desired amino acid sequence for homologous recombination in
35 accordance with well-known procedures.

In a still further aspect, the invention relates to a

recombinant expression vector which comprises a DNA construct of the invention. The recombinant expression vector may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence encoding the TFPI Kunitz-type domain II variant of the invention should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA encoding the TFPI Kunitz-type domain II variant of the invention in mammalian cells are the SV 40 promoter (Subramani et al., Mol. Cell Biol. 1, 1981, pp. 854-864), the MT-1 (metallothionein gene) promoter (Palmiter et al., Science 222, 1983, pp. 809-814) or the adenovirus 2 major late promoter. Suitable promoters for use in yeast host cells include promoters from yeast glycolytic genes (Hitzeman et al., J. Biol. Chem. 255, 1980, pp. 12073-12080; Alber and Kawasaki, J. Mol. Appl. Gen. 1, 1982, pp. 419-434) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals (Hollaender et al, eds.), Plenum Press, New York, 1982), or the TPI1 (US 4, 599, 311) or ADH2-4c (Russell et al., Nature 304, 1983, pp. 652-654) promoters. Suitable promoters for use in filamentous fungus host cells are, for instance, the ADH3 promoter (McKnight et al., The EMBO J. 4, 1985, pp. 2093-2099) or the tpiA promoter.

The DNA sequence encoding the TFPI Kunitz-type domain II variant

of the invention may also be operably connected to a suitable terminator, such as the human growth hormone terminator (Palmiter et al., op. cit.) or (for fungal hosts) the TPI1 (Alber and Kawasaki, op. cit.) or ADH3 (McKnight et al., op. cit.) promoters. The vector may further comprise elements such as polyadenylation signals (e.g. from SV 40 or the adenovirus 5 Elb region), transcriptional enhancer sequences (e.g. the SV 40 enhancer) and translational enhancer sequences (e.g. the ones encoding adenovirus VA RNAs).

10

The recombinant expression vector of the invention may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An examples of such a sequence (when the host cell is a mammalian cell) is the SV 40 origin of replication, or (when the host cell is a yeast cell) the yeast plasmid 2 μ replication genes REP 1-3 and origin of replication. The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or one which confers resistance to a drug, e.g. neomycin, hygromycin or methotrexate, or the Schizosaccharomyces pombe TPI gene (described by P.R. Russell, Gene 40, 1985, pp. 125-130).

The procedures used to ligate the DNA sequences coding for the TFPI Kunitz-type domain II variant of the invention, the promoter and the terminator, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, New York, 1989).

The host cell into which the expression vector of the invention is introduced may be any cell which is capable of producing the TFPI Kunitz-type domain II variant of the invention and is preferably a eukaryotic cell, such as a mammalian, yeast or fungal cell.

The yeast organism used as the host cell according to the invention may be any yeast organism which, on cultivation, produces large quantities of the TFPI Kunitz-type domain II variant of the invention. Examples of suitable yeast organisms are strains of the yeast species Saccharomyces cerevisiae, Saccharomyces kluyveri, Schizosaccharomyces pombe or Saccharomyces uvarum. The transformation of yeast cells may for instance be effected by protoplast formation followed by transformation in a manner known per se.

10

Examples of suitable mammalian cell lines are the COS (ATCC CRL 1650), BHK (ATCC CRL 1632, ATCC CCL 10) or CHO (ATCC CCL 61) cell lines. Methods of transfecting mammalian cells and expressing DNA sequences introduced in the cells are described in e.g. Kaufman and Sharp, J. Mol. Biol. 159, 1982, pp. 601-621; Southern and Berg, J. Mol. Appl. Genet. 1, 1982, pp. 327-341; Loyter et al., Proc. Natl. Acad. Sci. USA 79, 1982, pp. 422-426; Wigler et al., Cell 14, 1978, p. 725; Corsaro and Pearson, Somatic Cell Genetics 7, 1981, p. 603, Graham and van der Eb, Virology 52, 1973, p. 456; and Neumann et al., EMBO J. 1, 1982, pp. 841-845.

Alternatively, fungal cells may be used as host cells of the invention. Examples of suitable fungal cells are cells of filamentous fungi, e.g. Aspergillus spp. or Neurospora spp., in particular strains of Aspergillus oryzae or Aspergillus niger. The use of Aspergillus spp. for the expression of proteins is described in, e.g., EP 238 023.

30 The present invention further relates to a method of producing a TFPI Kunitz-type domain II variant according to the invention, the method comprising culturing a cell as described above under conditions conducive to the expression of the variant and recovering the resulting variant from the culture.

35

The medium used to cultivate the cells may be any conventional medium suitable for growing mammalian cells or fungal (including

yeast) cells, depending on the choice of host cell. The variant will be secreted by the host cells to the growth medium and may be recovered therefrom by conventional procedures including separating the cells from the medium by centrifugation or
5 filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulfate, purification by a variety of chromatographic procedures, e.g. ion exchange chromatography or affinity chromatography, or the like.

10

The present invention also relates to a pharmaceutical composition comprising a TFPI Kunitz-type domain II variant of the invention together with a pharmaceutically acceptable carrier or excipient. In the composition of the invention, the
15 variant may be formulated by any of the established methods of formulating pharmaceutical compositions, e.g. as described in Remington's Pharmaceutical Sciences, 1985. The composition may typically be in a form suited for systemic injection or infusion and may, as such, be formulated with sterile water or an
20 isotonic saline or glucose solution.

The TFPI Kunitz-type domain II variant of the invention is therefore contemplated to be advantageous to use for the therapeutic applications suggested for native aprotinin or
25 aprotinin analogues with other inhibitor profiles, in particular those which necessitate the use of large aprotinin doses. Therapeutic applications for which the use of the variant of the invention is indicated as a result of its ability to inhibit human serine proteases, e.g. trypsin, plasmin, kallikrein,
30 elastase, cathepsin G and proteinase-3, include (but are not limited to) acute pancreatitis, inflammation, thrombocytopenia, preservation of platelet function, organ preservation, wound healing, shock (including shock lung) and conditions involving hyperfibrinolytic haemorrhage, emphysema, rheumatoid arthritis,
35 adult respiratory distress syndrome, chronic inflammatory bowel disease and psoriasis, in other words diseases presumed to be caused by pathological proteolysis by elastase, cathepsin G and

proteinase-3 released from triggered PMNs.

Furthermore, the present invention relates to the use of TFPI Kunitz-type inhibitor domain II or a variant thereof as described above for the preparation of a medicament for the prevention or therapy of diseases or conditions associated with pathological proteolysis by proteases released from overstimulated PMNs. As indicated above, it may be an advantage of administer heparin concurrently with the TFPI Kunitz-type inhibitor domain II or variant.

Apart from the pharmaceutical use indicated above, TFPI Kunitz-type domain II or a variant thereof as specified above may be used to isolate useful natural substances, e.g. proteases or receptors from human material, which bind directly or indirectly to TFPI Kunitz-type domain II, for instance by screening assays or by affinity chromatography.

EXAMPLES

20

General methods.

Standard DNA techniques were carried out as described (Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). Synthetic oligonucleotides were prepared on an automatic DNA synthesizer (380B, Applied Biosystems) using phosphoramidite chemistry on a controlled pore glass support (Beaucage, S.L., and Caruthers, M.H., Tetrahedron Letters 22, (1981) 1859-1869). DNA sequence determinations were performed by the dideoxy chain-termination technique (Sanger, F., Micklen, S., and Coulson, A.R., Proc. Natl. Acad. Sci. USA 74 (1977) 5463-5467). Polymerase chain reactions (PCR) were performed on a DNA Thermal Cycler (Perkin Elmer Cetus)..

35

Amino acid analysis was carried out after hydrolysis in 6M HCl at 110°C in vacuum-sealed tubes for 24 hours. Analysis was

performed on a Beckman 121MB automatic amino acid analyzer modified for microbore operation.

5 N-terminal amino acid sequence analysis was obtained by automated Edman degradation using an Applied Biosystems 470A gas-phase sequencer. Analysis by on-line reverse phase HPLC was performed for the detection and quantitation of the liberated PTH amino acids from each sequencer cycle.

10 Molecular weight determination was obtained on a BIO-ION 20 plasma desorption mass spectrometer (PDMS) equipped with a flight tube of approximately 15 cm and operated in positive mode. Aliquots of 5 μ l were analyzed at an accelerating voltage set to 15 kV and ions were collected for 5 million fission
15 events. The accuracy on assigned molecular ions is approximately 0.1% for well defined peaks, otherwise somewhat less.

Example 1

20 Production of the second Kunitz domain of tissue factor pathway inhibitor, TFPI-2, from yeast strain KFN-1593.

cDNA encoding full length TFPI was isolated from the human liver derived cell line HepG2 (ATCC HB 8065) and inserted as a 0.9 kb
25 BamHI - XbaI fragment into a mammalian expression vector, pKFN-1168, as described (Pedersen, A.H., Nordfang, O., Norris, F., Wiberg, F.C., Christensen, P.M., Moeller, K.B., Meidahl-Pedersen, J., Beck, T.C., Norris, K., Hedner, U., and Kisiel, W. 1990, J. Biol. Chem. 265, 16786 - 16793). The DNA sequence of
30 the insert is given in SEQ ID No. 7. TFPI-2 is encoded by nucleotides 365-538 as indicated.

TFPI-2: 0.1 μ g of the 0.9 kb BamHI-XbaI fragment from pKFN-1168 was used as a template in a PCR reaction containing 100 pmole
35 each of the primers NOR-2526 (GCTGAGAGATTGGAGAAGAGAAAGCCAGAT-TTCTGCTT) and NOR-2528 (CTGGAATCTAGATTAACCATCTTCACAAATGTT). The 17 3'-terminal bases of NOR-2526 are identical to bases 365 to

381 in the TFPI-2 gene in SEQ ID No. 7, and the 21 5'-terminal bases are identical to bases 215 to 235 in the synthetic leader gene (see fig. 2) from pKFN-1000 described below. Primer NOR-2528 is complementary to bases 521 to 540 in SEQ ID No. 7 and has a 5' extension containing a translation stop codon followed by an XbaI site.

The PCR reaction was performed in a 100 μ l volume using a commercial kit (GeneAmp, Perkin Elmer Cetus) and the following cycle: 94° for 20 sec, 50° for 20 sec, and 72° for 30 sec. After 19 cycles a final cycle was performed in which the 72° step was maintained for 10 min. The PCR product, a 210 bp fragment, was isolated by electrophoresis on a 2 % agarose gel.

Signal-leader: 0.1 μ g of a 0.7 kb PvuII fragment from pKFN-1000 described below was used as a template in a PCR reaction containing 100 pmole each of the primers NOR-1478 (GTAAAC-GACGGCCAGT) and NOR-2523 (TCTCTTCTCCAATCTCTCAGC). NOR-1478 is matching a sequence just upstream of the EcoRI site in SEQ ID No. 9. Primer NOR-2523 is complementary to the 17 3'-terminal bases of the synthetic leader gene of pKFN-1000, see SEQ ID No. 9. The PCR reaction was performed as described above, resulting in a 257 bp fragment.

Plasmid pKFN-1000 is a derivative of plasmid pTZ19R (Mead, D.A., Szczesna-Skorupa, E. and Kemper, B., Prot. Engin. 1 (1986) 67-74) containing DNA encoding a synthetic yeast signal-leader peptide. Plasmid pKFN-1000 is described in WO 90/10075. The DNA sequence of 235 bp downstream from the EcoRI site of pKFN-1000 and the encoded amino acid sequence of the synthetic yeast signal-leader is given in SEQ ID No. 9.

Signal-leader-TFPI-2: Approx. 0.1 μ g of each of the two PCR-fragments described above were mixed. A PCR reaction was performed using 100 pmole each of primers NOR-1478 and NOR-2528 and the following cycle: 94° for 1 min, 50° for 2 min, and 72° for 3 min. After 16 cycles a final cycle was performed in which

the 72° step was maintained for 10 min.

The resulting 442 bp fragment was purified by electrophoresis on a 1 % agarose gel and then digested with EcoRI and XbaI. The resulting 412 bp fragment was ligated to the 9.5 kb NcoI-XbaI fragment from pMT636 and the 1.4 kb NcoI-EcoRI fragment from pMT636. Plasmid pMT636 is described in International Patent application No. PCT/DK88/00138.

10 pMT636 is an *E. coli* - *S. cerevisiae* shuttle vector containing the *Schizosaccharomyces pombe* TPI gene (POT) (Russell, P.R., *Gene* 40 (1985) 125-130), the *S. cerevisiae* triosephosphate isomerase promoter and terminator, TPI_p and TPI_t (Alber, T., and Kawasaki, G. *J. Mol. Appl. Gen.* 1 (1982), 419-434).

15 The ligation mixture was used to transform a competent *E. coli* strain (r⁻, m⁺) selecting for ampicillin resistance. DNA sequencing showed that plasmids from the resulting colonies contained the correct DNA sequence for TFPI-2 correctly fused to the synthetic yeast signal-leader gene.

One plasmid pKFN-1605 was selected for further use. The construction of plasmid pKFN-1605 is illustrated in Fig. 1.

25 The expression cassette of plasmid pKFN-1605 contains the following sequence:

TPI_p - KFN1000 signal-leader - TFPI2 - TPI_t

The DNA sequence of the 412 bp EcoRI-XbaI fragment from pKFN-1605 is shown in SEQ ID No. 11.

30 Yeast transformation: *S. cerevisiae* strain MT663 (E2-7B XE11-36 a/α, Δtpi/Δtpi, pep 4-3/pep 4-3) was grown on YPGaL (1% Bacto yeast extract, 2% Bacto peptone, 2% galactose, 1% lactate) to an O.D. at 600 nm of 0.6.

100 ml of culture was harvested by centrifugation, washed with

10 ml of water, recentrifuged and resuspended in 10 ml of a solution containing 1.2 M sorbitol, 25 mM Na₂EDTA pH = 8.0 and 6.7 mg/ml dithiotreitol. The suspension was incubated at 30°C for 15 minutes, centrifuged and the cells resuspended in 10 ml of a solution containing 1.2 M sorbitol, 10 mM Na₂EDTA, 0.1 M sodium citrate, pH = 5.8, and 2 mg Novozym® 234. The suspension was incubated at 30°C for 30 minutes, the cells collected by centrifugation, washed in 10 ml of 1.2 M sorbitol and 10 ml of CAS (1.2 M sorbitol, 10 mM CaCl₂, 10 mM Tris HCl (Tris = Tris(hydroxymethyl)aminomethane) pH = 7.5) and resuspended in 2 ml of CAS. For transformation, 0.1 ml of CAS-resuspended cells were mixed with approx. 1 µg of plasmid pKFN-1605 and left at room temperature for 15 minutes. 1 ml of (20% polyethylene glycol 4000, 20 mM CaCl₂, 10 mM CaCl₂, 10 mM Tris HCl, pH = 7.5) was added and the mixture left for a further 30 minutes at room temperature. The mixture was centrifuged and the pellet resuspended in 0.1 ml of SOS (1.2 M sorbitol, 33% v/v YPD, 6.7 mM CaCl₂, 14 µg/ml leucine) and incubated at 30°C for 2 hours. The suspension was then centrifuged and the pellet resuspended in 0.5 ml of 1.2 M sorbitol. Then, 6 ml of top agar (the SC medium of Sherman et al., (Methods in Yeast Genetics, Cold Spring Harbor Laboratory (1982)) containing 1.2 M sorbitol plus 2.5% agar) at 52°C was added and the suspension poured on top of plates containing the same agar-solidified, sorbitol containing medium.

Transformant colonies were picked after 3 days at 30°C, reisolated and used to start liquid cultures. One such transformant KFN-1593 was selected for further characterization.

30

Fermentation: Yeast strain KFN-1593 was grown on YPD medium (1% yeast extract, 2% peptone (from Difco Laboratories), and 3% glucose). A 1 liter culture of the strain was shaken at 30°C to an optical density at 650 nm of 24. After centrifugation the supernatant was isolated.

35

Purification: The yeast supernatant (1000 ml) adjusted to pH 3.0 with phosphoric acid was applied on a column of S-Sepharose Fast Flow (Pharmacia, 2.6 x 3.6 cm) equilibrated with 25 mM sodium dihydrogen phosphate, pH = 3.5. After wash with equilibration
5 buffer, TFPI-2 assayed as trypsin inhibitory activity was eluted with buffer containing 1M sodium chloride (40 ml). Desalting was obtained on a Sephadex G-25 column (Pharmacia, 2.6 x 34 cm) equilibrated and eluted with ammonium hydrogen carbonate, pH = 7.5. Further purification was performed on a Mono S column
10 (Pharmacia, 0.5 x 5 cm) by gradient elution over 23 min at 1 ml/min from 0 - 0.43 M sodium chloride in 25 mM sodium dihydrogen phosphate, 10% w/v acetonitrile, pH = 3.5. N-glycosylated TFPI-2 and unglycosylated TFPI-2 eluted at 0.20 M and 0.26 M sodium chloride, respectively. Final purification of
15 unglycosylated TFPI-2 was performed by reverse phase HPLC on a C18 column (Novo Nordisk A/S, 0.4 x 25 cm) by gradient elution over 30 min at 1 ml/min from 0 - 50% acetonitrile, 0.1% trifluoroacetic acid.

20 TFPI-2 eluted at 40% acetonitrile. The purified product was lyophilized and redissolved in water to a concentration of approx. 200 nM. Aliquot samples of this solution were analysed for amino acid composition (table 1), amino acid sequence, molecular weight (PDMS, found: MW 6840.8, calc.: 6840.6) and
25 protease inhibitory activities.

Example 2

Production of [R15K, G16A, Y17R, T19I]-TFPI-2 from yeast strain
30 KFN-1811.

0.1 µg of the 1.3 kb SphI-BamHI fragment encoding TFPI-2 from plasmid pKFN-1605 was used as a template in two PCR reactions. In the first PCR reaction 100 pmole each of the primers NOR-2022
35 (GGAGTTTAGTGAAGTTGC) and M-460 (GTTATAAAAATACCTGATAATACGAGCTT-TACATATTCCAGGATC) was used. In the second PCR reaction 100 pmole each of the primers NOR-1495 (TAAGTGGCTCAGAATGA) and M-459 (GAT-

CCTGGAATATGTAAAGCTCGTATTATCAGGTATTTTATAAC) was used.

NOR-2022 primes at a position 94 bp downstream of the SphI site. M-460 is complementary to the TFPI-2 DNA-sequence position 263-307, SEQ ID No. 11, except for six mismatches. NOR-1495 primes at a position 561 bp upstream from the BamHI site. M-459 is complementary to M-460.

The PCR reaction was performed in a 100 μ l volume using a commercial kit (GeneAmp, Perkin Elmer Cetus) and the following cycle: 95° for 1 min, 50° for 1 min, and 72° for 2 min. After 24 cycles a final cycle was performed in which the 72° step was maintained for 10 min. The PCR products, a 444 bp fragment from the first PCR and a 285 bp fragment from the second, were isolated by electrophoresis on a 2 % agarose gel.

Approx. 0.1 μ g of each of the two PCR-fragments described above were mixed. A PCR reaction was performed using 100 pmole each of primers NOR-2022 and NOR-1495 and the following cycle: 95° for 1 min, 50° for 2 min, and 72° for 3 min. After 22 cycles a final cycle was performed in which the 72° step was maintained for 10 min.

The resulting 687 bp fragment was purified by electrophoresis on a 1 % agarose gel and then digested with EcoRI and XbaI. The resulting 412 bp fragment was ligated to the 9.5 kb NcoI-XbaI fragment from pMT636 and the 1.4 kb NcoI-EcoRI fragment from pMT636. Plasmid pMT636 is described in example 1.

The ligation mixture was used to transform a competent *E. coli* strain r⁻, m⁺) selecting for ampicillin resistance. DNA sequencing showed that plasmids from the resulting colonies contained the correct DNA sequence for [R15K, G16A, Y17R, T19I]-TFPI-2 fused to the synthetic yeast signal-leader gene.

One plasmid pKFN-1798 was selected for further use. The DNA sequence of the 412 bp EcoRI-XbaI fragment from pKFN-1798 is shown in SEQ ID No. 13.

Plasmid pKFN-1798 was transformed in yeast strain MT663 as described in example 1 resulting in yeast strain KFN-1811.

5 Culturing of the transformed strain KFN-1811 in YPD-medium, analysis for [R15K, G16A, Y17R, T19I]-TFPI-2 in the supernatant, and purification was performed as described in example 1.

Example 3

10 Inhibition of serine proteinases by TFPI (domain II) KFN 1593

KFN 1593 was purified from yeast culture medium as described in example 1. The concentration of KFN 1593 was determined using 1% $E_{280\text{ nm}} = 8.3$ and $M_r = 6500$. Porcine trypsin was from Novo Nordisk
15 (Bagsværd, Denmark), bovine chymotrypsin (TLCK treated) and porcine pancreatic kallikrein was from Sigma Chemical Co (St. Louis, MO, USA), human plasmin and human plasma kallikrein was from Kabi (Stockholm, Sweden).

20 Human neutrophil elastase and cathepsin G was purified from extracts of PMNs according to the method described by Baugh and Travis (Biochemistry 15 (1976) 836-843). Peptidyl nitroanilide substrates, S2251, S2586, S2266, S2302 were from Kabi
25 (Stockholm, Sweden). M4765 and S7388 was from Sigma Chemical Co (St. Louis, MO, USA) and FXa-1 was from NycoMed (Oslo, Norway).

Serine proteinases were incubated with various concentrations of KFN 1593 for 30 min. Substrate was then added and residual proteinase activity was measured at 405 nm. The results are
30 shown in Fig. 2 and Fig. 3.

Unmodified TFPI Kunitz domain II (KFN 1593) is an inhibitor of trypsin ($K_i = 5 \times 10^{-9}\text{M}$) and factor X. ($K_i = 150\text{ nM}$). KFN 1593 shows a moderate inhibition of plasmin and neutrophil elastase,
35 whereas the inhibition of Cathepsin G and kallikreins is essentially absent.

Table 1

	Amino acid	TFPI-2	
		Theor.	Found
5	Ala	0	0.31
	Cys	6	5.14
	Asx	9	8.94
	Glx	9	9.25
10	Phe	5	4.89
	Gly	6	6.01
	His	0	0.13
	Ile	3	2.82
	Lys	4	4.12
15	Leu	3	3.04
	Met	1	0.82
	Pro	2	2.08
	Arg	3	2.86
	Ser	0	0.17
20	Thr	3	2.92
	Val	0	0.16
	Trp	0	-
	Tyr	4	3.75
25	Total	58	57.41

Example 4

Production of [R15K, G16A, Y17R, T19I, L39R]-TFPI-2 from yeast strain KFN-1867.

30

0.1 μ g of the 1.3 kb SphI-BamHI fragment encoding [R15K, G16A, Y17R, T19I]-TFPI-2 from plasmid pKFN-1798 was used as a template in two PCR reactions. In the first PCR reaction 100 pmole each of the primers NOR-2022 (GGAGTTTAGTGAAGTTGC) and M-462 (CCAG-TGTCTCAAAATTGTTTCATATTGCCCTGCATCCACC) was used. In the second PCR reaction 100 pmole each of the primers NOR-1495 (TAAGTGGCTCAGAA-TGA) and M-461 (GGTGGATGCAGGGGCAATATGAACAATTTTGAGACACTGG) was used.

35

NOR-2022 primes at a position 94 bp downstream of the SphI site. M-462 is complementary to the TFPI-2 DNA-sequence position 341-380, SEQ ID No. 11, except for two mismatches. NOR-1495 primes at a position 561 bp upstream from the BamHI site. M-461 is complementary to M-462.

40

45 The PCR reaction was performed in a 100 μ l volume using a

commercial kit (GeneAmp, Perkin Elmer Cetus) and the following cycle: 95° for 1 min, 50° for 1 min, and 72° for 2 min. After 24 cycles a final cycle was performed in which the 72° step was maintained for 10 min. The PCR products, a 518 bp fragment from
5 the first PCR and a 209 bp fragment from the second, were isolated by electrophoresis on a 2 % agarose gel.

Approx. 0.1 µg of each of the two PCR-fragments described above were mixed. A PCR reaction was performed using 100 pmole each of primers NOR-2022 and NOR-1495 and the following cycle: 95° for
10 1 min, 50° for 2 min, and 72° for 3 min. After 22 cycles a final cycle was performed in which the 72° step was maintained for 10 min.

The resulting 687 bp fragment was purified by electrophoresis on
15 a 1 % agarose gel and then digested with EcoRI and XbaI. The resulting 412 bp fragment was ligated to the 9.5 kb NcoI-XbaI fragment from pMT636 and the 1.4 kb NcoI-EcoRI fragment from pMT636. Plasmid pMT636 is described in example 1.

20 The ligation mixture was used to transform a competent E. coli strain r⁻, m⁺) selecting for ampicillin resistance. DNA sequencing showed that plasmids from the resulting colonies contained the correct DNA sequence for [R15K, G16A, Y17R, T19I, L39R]-TFPI-2 fused to the synthetic yeast signal-leader gene.

25 One plasmid pKFN-1861 was selected for further use. The DNA sequence of the 412 bp EcoRI-XbaI fragment from pKFN-1861 is shown in SEQ ID No. 15.

30 Plasmid pKFN-1861 was transformed in yeast strain MT663 as described in example 1 resulting in yeast strain KFN-1867.

Culturing of the transformed strain KFN-1867 in YPD-medium, analysis for [R15K, G16A, Y17R, T19I, L39R]-TFPI-2 in the
35 supernatant, and purification was performed as described in example 1.

Example 5

Production of [R15K, G16A, Y17R, T19I, L39R, E46K]-TFPI-2 from yeast strain KFN-1868.

5 0.1 μ g of the 1.3 kb SphI-BamHI fragment encoding [R15K, G16A, Y17R, T19I]-TFPI-2 from plasmid pKFN-1798 was used as a template in two PCR reactions. In the first PCR reaction 100 pmole each of the primers NOR-2022 (GGAGTTTAGTGAAGTTGC) and M-464 (CCAG-TGTCTTAAAATTGTTTCATATTGCCCTGCATCCACC) was used. In the second
10 PCR reaction 100 pmole each of the primers NOR-1495 (TAAGTGGCTCAGAA-TGA) and M-463 (GGTGGATGCAGGGGCAATATGAACAATTTTAAGACACTGG) was used. NOR-2022 primes at a position 94 bp downstream of the SphI site. M-464 is complementary to the TFPI-2 DNA-sequence position 341-
15 380, SEQ ID No. 11, except for three mismatches. NOR-1495 primes at a position 561 bp upstream from the BamHI site. M-463 is complementary to M-464.

The PCR reaction was performed in a 100 μ l volume using a
20 commercial kit (GeneAmp, Perkin Elmer Cetus) and the following cycle: 95° for 1 min, 50° for 1 min, and 72° for 2 min. After 24 cycles a final cycle was performed in which the 72° step was maintained for 10 min. The PCR products, a 518 bp fragment from the first PCR and a 209 bp fragment from the second, were
25 isolated by electrophoresis on a 2 % agarose gel. Approx. 0.1 μ g of each of the two PCR-fragments described above were mixed. A PCR reaction was performed using 100 pmole each of primers NOR-2022 and NOR-1495 and the following cycle: 95° for 1 min, 50° for 2 min, and 72° for 3 min. After 22 cycles a final
30 cycle was performed in which the 72° step was maintained for 10 min.

The resulting 687 bp fragment was purified by electrophoresis on a 1 % agarose gel and then digested with EcoRI and XbaI. The
35 resulting 412 bp fragment was ligated to the 9.5 kb NcoI-XbaI fragment from pMT636 and the 1.4 kb NcoI-EcoRI fragment from pMT636. Plasmid pMT636 is described in example 1.

The ligation mixture was used to transform a competent *E. coli* strain r⁻, m⁺) selecting for ampicillin resistance. DNA sequencing showed that plasmids from the resulting colonies contained the correct DNA sequence for [R15K, G16A, Y17R, T19I, L39R, E46K]-TFPI-2 fused to the synthetic yeast signal-leader gene.

One plasmid pKFN-1862 was selected for further use. The DNA sequence of the 412 bp EcoRI-XbaI fragment from pKFN-1862 is shown in SEQ ID No. 17.

Plasmid pKFN-1862 was transformed in yeast strain MT663 as described in example 1 resulting in yeast strain KFN-1868.

Culturing of the transformed strain KFN-1868 in YPD-medium, analysis for [R15K, G16A, Y17R, T19I, L39R, E46K]-TFPI-2 in the supernatant, and purification was performed as described in example 1.

Example 6

Multiple mutation of TFPI-2 in position 15 and 16.

0.1 µg of the 1.3 kb SphI-BamHI fragment encoding TFPI-2 from plasmid pKFN-1605 was used as a template in two PCR reactions. In the first PCR reaction 100 pmole each of the primers NOR-2022 (GGAGTTTAGTGAAGTTGC) and M-749 (AATACCTGGTAATATAA(C/G)C(C/G)A-(A/C)ACATATTCCAGGATC) was used. In the second PCR reaction 100 pmole each of the primers NOR-1495 (TAAGTGGCTCAGAATGA) and M-750 (GATCCTGGAATATGT(T/G)T(C/G)G(C/G)TTATATTACCAGGTATT) was used. NOR-2022 primes at a position 94 bp downstream of the SphI site. M-749 is complementary to the TFPI-2 DNA-sequence position 263-299, SEQ ID No. 11, except for four mismatches. NOR-1495 primes at a position 561 bp upstream from the BamHI site. M-750 is complementary to M-749.

35

The PCR reaction was performed in a 100µl volume using a commercial kit (GeneAmp, Perkin Elmer Cetus) and the following

cycle: 95° for 1 min, 50° for 1 min, and 72° for 2 min. After 24 cycles a final cycle was performed in which the 72° step was maintained for 10 min. The PCR products, a 439 bp fragment from the first PCR and a 285 bp fragment from the second, were
 5 isolated by electrophoresis on a 2 % agarose gel.
 Approx. 0.1 µg of each of the two PCR-fragments described above were mixed. A PCR reaction was performed using 100 pmole each of primers NOR-2022 and NOR-1495 and the following cycle: 95° for 1 min, 50° for 2 min, and 72° for 3 min. After 22 cycles a final
 10 cycle was performed in which the 72° step was maintained for 10 min.

The resulting 687 bp fragment was purified by electrophoresis on a 1 % agarose gel and then digested with EcoRI and XbaI. The
 15 resulting 412 bp fragment was ligated to the 2.8 kb EcoRI-XbaI fragment from plasmid pTZ19R (Mead, D. A., Szczesna-Skopura, E., and Kemper, B. Prot. Engin. 1 (1986) 67-74).

The ligation mixture was used to transform a competent E. coli
 20 strain r⁻, m⁺) selecting for ampicillin resistance. By DNA sequencing the following six plasmids encoding the indicated TFPI-2 analogs fused to the synthetic yeast signal-leader gene were identified:

	Plasmid	Analog
25	pKFN-1885	[R15F]-TFPI-2
	pKFN-1883	[R15F, G16A]-TFPI-2
	pKFN-1905	[R15L]-TFPI-2
	pKFN-1882	[R15L, G16A]-TFPI-2
30	pKFN-1887	[R15V]-TFPI-2
	pKFN-1886	[R15V, G16A]-TFPI-2

The 412 bp EcoRI-XbaI fragments from these plasmids were used for the construction of the expression plasmids as described in
 35 example 1.

Transformation of yeast strain MT-663 as described in example 1

resulted in the following yeast strains:

	<u>Yeast strain</u>	<u>Analog</u>
	KFN-1896	[R15F]-TFPI-2
5	KFN-1894	[R15F, G16A]-TFPI-2
	KFN-1928	[R15L]-TFPI-2
	KFN-1893	[R15L, G16A]-TFPI-2
	KFN-1898	[R15V]-TFPI-2
	KFN-1897	[R15V, G16A]-TFPI-2

10

Culturing of the transformed yeast strains in YPD-medium, analysis for TFPI-2 analogs in the supernatant, and purification was performed as described in example 1.

15 Example 8

Inhibition of serine proteinases by TFPI (domain II) KFN 1811, 1867 and 1868

20 The three TFPI (domain II) variants were purified from yeast culture medium. Their concentrations were determined from the absorbance at 214 nm using BPTI as a standard. The final concentration was determined by titration with trypsin. Porcine trypsin and human recombinant proteins, factor VIIa, activated
 25 protein C (ACP), and tPA were obtained from Novo Nordisk A/S (Bagsvaerd, Denmark), so was human thrombin. Bovine chymotrypsin and glandular kallikrein were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Truncated human recombinant tissue factor
 30 cathepsin G was purified from extracts of PMNs according to the method described by Baugh and Travis (Biochemistry 15 (1976) 836-843). Human plasmin was from Kabi (Stockholm, Sweden), uPA was from Serono (Milan, Italy), human factor Xa was a gift from Dr. W. Kisiel (Albuquerque, NM, USA), and human plasma
 35 kallikrein was a gift from Dr. I Schousboe (Copenhagen, Denmark).

Peptidyl nitroanilide substrates, S2251, S2302, S2266, S2586, S2288, S2444, S2366, and S2238 were from Kabi (Stockholm, Sweden). S7388 and M4765 were from Sigma Chemical Co. (St. Louis, MO, USA) and FXa-1 was from Nycomed (Oslo, Norway).

5

Serine proteinases were incubated with various concentrations of Kunitz Domain variant for 30 min. Substrate (0.6 mM) was then added and residual proteinase activity was measured at 405 nm. The results are shown in Table 1.

10

The three variants are strong specific plasmin inhibitors without significant inhibition of other proteinases from plasma tested.

Table 1

Enzyme	Enzyme Conc.	Substrate	Apparant K_i (nM)		
			KFN 1811	KFN 1867	KFN 1868
Trypsin	10 nM	S2251	<<1	<<1	<<1
Plasmin	4 nM	S2251	3	3	3
N. elastase	10 nM	M4765	n.i.	n.i.	n.i.
N. cathepsin G	50 nM	S7388	n.i.	n.i.	n.i.
Pl. kallikrein	3 nM	S2302	>100	>100	>100
Gl. kallikrein	1 U/ml	S2266	>100	>100	>100
chymotrypsin	2.5 nM	S2586	10	20	20
tPA	10 nM	S2288	n.i.	n.i.	n.i.
Factor VIIa/TF	10 nM/15 nM	FXa-1	n.i.	n.i.	n.i.
Factor Xa	3 nM	FXa-1	n.i.	n.i.	n.i.
uPA	5 nM	S2444	n.i.	n.i.	n.i.
APC	5 nM	S2366	n.i.	n.i.	n.i.
Thrombin	3 NIHu/ml	S2238	n.i.	n.i.	n.i.

Example 9Inhibition of serine proteinases by TFPI (domain II) KFN 1893, 1897, 1898 and 1928

The four variants were purified from yeast culture medium. Their
5 concentrations were determined from the absorbance at 214 nm
using BTPI as a standard. Porcine trypsin was obtained from Novo
Nordisk A/S (Bagsvaerd, Denmark, bovine chymotrypsin (TLCK
treated) was obtained from Sigma Chemical Co. (St. Louis, MO,
USA). Truncated human recombinant tissue factor was obtained
10 from Corvas (San Diego, CA, USA). Human plasmin was from Kabi
(Stockholm, Sweden). Human neutrophil cathepsin G and elastase
were purified from extracts of PMNs according to the method
described by Baugh and Travis (Biochemistry 15 (1976) 836-843).

Peptidyl nitroanilide substrates, S2251, S2586 were from Kabi
15 (Stockholm, Sweden). S7388 and M4765 were from Sigma Chemical
Co. (St. Louis, MO, USA).

Serine proteinases were incubated with various concentrations of
the variants for 30 min. Substrate (0.6 nM) was then added and
residual proteinase activity was measured at 405 nm. The results
20 are shown in Table 2.

The four TFPI Kunitz domain II variants (KFN 1893, 1897, 1898,
1928) were found to be strong inhibitors of neutrophil elastase.

Table 2

Enzyme	Enzyme Conc.	Substrate	Apparant K _i (nM)			
			KNN 1893	KFN 1897	KFN 1898	KFN 1928
Trypsin	10 nM	S2251	n.i.	n.i.	n.i.	n.i.
Chymotrypsin	2.5 nM	S2586	<5	w.i.	w.i.	<5
N. elastase	4 nM	M4765	0.23	0.46	0.35	2.2
N. cathepsin G	50 nM	S7388	n.i.	n.i.	n.i.	n.i.
Plasmin	4 nM	S2251	n.i.	n.i.	n.i.	n.i.

n.i. No. inhibition at conc. <1 μM; w.i. weak inhibition at 100 nM.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Novo Nordisk A/S
- (B) STREET: Novo Alle
- (C) CITY: Bagsvaerd
- (E) COUNTRY: Denmark
- (F) POSTAL CODE (ZIP): DK-2880
- (G) TELEPHONE: +45 4444 8888
- (H) TELEFAX: +45 4449 3256
- (I) TELEX: 37304

(ii) TITLE OF INVENTION: A Human Kunitz-Type Protease Inhibitor Variant

(iii) NUMBER OF SEQUENCES: 18

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 57 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Xaa	Asp	Phe	Cys	Phe	Leu	Glu	Glu	Asp	Xaa	Gly	Xaa	Cys	Xaa	Xaa	Xaa	1	5	10	15
Xaa	Xaa	Xaa	Tyr	Phe	Tyr	Asn	Asn	Gln	Thr	Lys	Gln	Cys	Glu	Arg	Phe	20	25	30	
Xaa	Tyr	Gly	Gly	Cys	Xaa	Xaa	Xaa	Met	Asn	Asn	Phe	Xaa	Thr	Leu	Glu	35	40	45	
Glu	Cys	Lys	Asn	Ile	Cys	Glu	Asp	Xaa								50	55		

(2) INFORMATION FOR SEQ ID NO: 2:

32

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 57 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```

Xaa Asp Phe Cys Phe Leu Glu Glu Asp Xaa Gly Xaa Cys Xaa Xaa Xaa
1           5           10           15
Xaa Xaa Xaa Tyr Phe Tyr Asn Xaa Gln Xaa Lys Gln Cys Glu Arg Phe
                20           25           30
Xaa Tyr Gly Gly Cys Xaa Xaa Xaa Met Asn Asn Phe Xaa Thr Leu Glu
                35           40           45
Glu Cys Lys Asn Ile Cys Glu Asp Xaa
                50           55

```

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 58 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```

Lys Pro Asp Phe Cys Phe Leu Glu Glu Asp Pro Gly Ile Cys Lys Ala
1           5           10           15
Arg Ile Thr Arg Tyr Phe Tyr Asn Asn Gln Thr Lys Gln Cys Glu Arg
                20           25           30
Phe Lys Tyr Gly Gly Cys Leu Gly Asn Met Asn Asn Phe Glu Thr Leu
                35           40           45
Glu Glu Cys Lys Asn Ile Cys Glu Asp Gly
                50           55

```

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 58 amino acids

33

(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```

Lys Pro Asp Phe Cys Phe Leu Glu Glu Asp Pro Gly Ile Cys Lys Ala
1           5           10           15
Arg Ile Thr Arg Tyr Phe Tyr Asn Asn Gln Thr Lys Gln Cys Glu Arg
                20           25           30
Phe Lys Tyr Gly Gly Cys Arg Gly Asn Met Asn Asn Phe Glu Thr Leu
          35           40           45
Glu Glu Cys Lys Asn Ile Cys Glu Asp Gly
          50           55

```

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 58 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```

Lys Pro Asp Phe Cys Phe Leu Glu Glu Asp Pro Gly Ile Cys Lys Ala
1           5           10           15
Arg Ile Thr Arg Tyr Phe Tyr Asn Asn Gln Thr Lys Gln Cys Glu Arg
                20           25           30
Phe Lys Tyr Gly Gly Cys Arg Gly Asn Met Asn Asn Phe Lys Thr Leu
          35           40           45
Glu Glu Cys Lys Asn Ile Cys Glu Asp Gly
          50           55

```

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 58 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

34

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

```

Lys Pro Asp Phe Cys Phe Leu Glu Glu Asp Pro Gly Pro Cys Lys Ala
 1           5           10           15
Arg Ile Ile Arg Tyr Phe Tyr Asn Asn Gln Thr Lys Gln Cys Glu Arg
          20           25           30
Phe Val Tyr Gly Gly Cys Arg Ala Lys Met Asn Asn Phe Lys Thr Leu
          35           40           45
Glu Glu Cys Lys Asn Ile Cys Glu Asp Gly
          50           55

```

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 945 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 365..538

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

```

GGATCOGAAT TCCACCATGA AGAAAGTACA TGCACCTTGG GCTTCGTGAT GCCTGCTGCT      60
TAATCTTGCC CCTGCCCCCTC TTAATGCTGA TTCTGAGGAA GATGAAGAAC ACACAATTAT      120
CACAGATAAG GAGTTGCCAC CACTGAAACT TATGCATTCA TTTTGIGCAT TCAAGGOGGA      180
TGATGGCCCA TGTAAGCAA TCATGAAAAG ATTTTCTTTC AATATTTTCA CTOGACAGTG      240
OGAAGAATTT ATATATGGGG GATGTGAAGG AAATCAGAAT CGATTTGAAA GTCTGGAAGA      300
GTGCAAAAAA ATGIGTACAA GAGATAATGC AAACAGGATT ATAAAGACAA CATIGCAACA      360
AGAA AAG CCA GAT TTC TGC TTT TIG GAA GAA GAT CCT GGA ATA TGT CGA      409
Lys Pro Asp Phe Cys Phe Leu Glu Glu Asp Pro Gly Ile Cys Arg
 1           5           10           15

```

35

GGT TAT ATT ACC AGG TAT TTT TAT AAC AAT CAG ACA AAA CAG TGT GAA 457
 Gly Tyr Ile Thr Arg Tyr Phe Tyr Asn Asn Gln Thr Lys Gln Cys Glu
 20 25 30

CGT TTC AAG TAT GGT GGA TGC CTG GGC AAT ATG AAC AAT TTT GAG ACA 505
 Arg Phe Lys Tyr Gly Gly Cys Leu Gly Asn Met Asn Asn Phe Glu Thr
 35 40 45

CTG GAA GAA TGC AAG AAC ATT TGT GAA GAT GGT CCGAATGGTT TCCAGGTGGA 558
 Leu Glu Glu Cys Lys Asn Ile Cys Glu Asp Gly
 50 55

TAATTATGGA ACCCAGCTCA ATGCTGTGAA TAACTCCCTG ACTCOGCAAT CAACCAAGGT 618

TCCCAGCCTT TTTGAATTTC ACGGTCCCTC ATGGTGTCTC ACTCCAGCAG ACAGAGGATT 678

GTGTGTGTC AATGAGAACA GATTCTACTA CAATTCAGTC ATTGGGAAAT GCGGCCCAT 738

TAAGTACAGT GGATGTGGGG GAAATGAAAA CAATTTTACT TCCAAACAAG AATGTCCTGAG 798

GGCATGTAAA AAAGGTTTCA TCCAAAGAAT ATCAAAAGGA GGCTTAATTA AAACCAAAAG 858

AAAAAGAAAG AAGCAGAGAG TGAAAATAGC ATATGAAGAG ATCTTTTGTTA AAAATATGTG 918

AATTGTATAT AGCAATGTAA CTCTAGA 945

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 58 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Lys Pro Asp Phe Cys Phe Leu Glu Glu Asp Pro Gly Ile Cys Arg Gly
 1 5 10 15

Tyr Ile Thr Arg Tyr Phe Tyr Asn Asn Gln Thr Lys Gln Cys Glu Arg
 20 25 30

Phe Lys Tyr Gly Gly Cys Leu Gly Asn Met Asn Asn Phe Glu Thr Leu
 35 40 45

Glu Glu Cys Lys Asn Ile Cys Glu Asp Gly
 50 55

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 235 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

36

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: synthetic

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 77..235

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GAATTCATT CAAGAATAGT TCAAACAAGA AGATTACAAA CTATCAATTT CATAACAAT	60
ATAAAGGACC AAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC	109
Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile	
1 5 10	
GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG	157
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu	
15 20 25	
ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC	205
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn	
30 35 40	
GTC GCC ATG GCT GAG AGA TTG GAG AAG AGA	235
Val Ala Met Ala Glu Arg Leu Glu Lys Arg	
45 50	

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 53 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala	15
1 5 10	
Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser	30
20 25	
Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Glu	45
35 40	
Arg Leu Glu Lys Arg	50

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 418 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: synthetic/human

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 77..409

(ix) FEATURE:

- (A) NAME/KEY: sig_peptide
- (B) LOCATION: 77..235

(ix) FEATURE:

- (A) NAME/KEY: mat_peptide
- (B) LOCATION: 236..409

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GAATTCATT CAAGAATAGT TCAAACAAGA AGATTACAAA CTATCAATTT CATAACAAT	60
ATAAAGACC AAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC	109
Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile	
-53 -50 -45	
GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG	157
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu	
-40 -35 -30	
ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC	205
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn	
-25 -20 -15	
GTC GCC ATG GCT GAG AGA TTG GAG AAG AGA AAG CCA GAT TTC TGC TTT	253
Val Ala Met Ala Glu Arg Leu Glu Lys Arg Lys Pro Asp Phe Cys Phe	
-10 -5 1 5	
TTG GAA GAA GAT CCT GGA ATA TGT CGA GGT TAT ATT ACC AGG TAT TTT	301
Leu Glu Glu Asp Pro Gly Ile Cys Arg Gly Tyr Ile Thr Arg Tyr Phe	
10 15 20	
TAT AAC AAT CAG ACA AAA CAG TGT GAA CGT TTC AAG TAT GGT GGA TGC	349
Tyr Asn Asn Gln Thr Lys Gln Cys Glu Arg Phe Lys Tyr Gly Gly Cys	
25 30 35	

38

CTG GGC AAT ATG AAC AAT TTT GAG ACA CTG GAA GAA TGC AAG AAC ATT
 Leu Gly Asn Met Asn Asn Phe Glu Thr Leu Glu Glu Cys Lys Asn Ile
 40 45 50

397

TGT GAA GAT GGT TAATCTAGA
 Cys Glu Asp Gly
 55

418

(2) INFORMATION FOR SEQ ID NO: 12:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 111 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
 -53 -50 -45 -40
 Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
 -35 -30 -25
 Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Glu
 -20 -15 -10
 Arg Leu Glu Lys Arg Lys Pro Asp Phe Cys Phe Leu Glu Glu Asp Pro
 -5 1 5 10
 Gly Ile Cys Arg Gly Tyr Ile Thr Arg Tyr Phe Tyr Asn Asn Gln Thr
 15 20 25
 Lys Gln Cys Glu Arg Phe Lys Tyr Gly Gly Cys Leu Gly Asn Met Asn
 30 35 40
 Asn Phe Glu Thr Leu Glu Glu Cys Lys Asn Ile Cys Glu Asp Gly
 45 50 55

(2) INFORMATION FOR SEQ ID NO: 13:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 418 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
 (A) ORGANISM: synthetic

(ix) FEATURE:
 (A) NAME/KEY: CDS

(B) LOCATION: 77..409

(ix) FEATURE:

(A) NAME/KEY: sig peptide

(B) LOCATION: 77..235

(ix) FEATURE:

(A) NAME/KEY: mat_peptide

(B) LOCATION: 236..409

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GAATTCATT CAAGAATAGT TCAAACAAGA AGATTACAAA CTATCAATTT CATAACAAT	60
ATAAAGGACC AAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC	109
Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile	
-53 -50 -45	
GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG	157
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu	
-40 -35 -30	
ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC	205
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn	
-25 -20 -15	
GTC GCC ATG GCT GAG AGA TTG GAG AAG AGA AAG CCA GAT TTC TGC TTT	253
Val Ala Met Ala Glu Arg Leu Glu Lys Arg Lys Pro Asp Phe Cys Phe	
-10 -5 1 5	
TTG GAA GAA GAT CCT GGA ATA TGT AAA GCT CGT ATT ATC AGG TAT TTT	301
Leu Glu Glu Asp Pro Gly Ile Cys Lys Ala Arg Ile Ile Arg Tyr Phe	
10 15 20	
TAT AAC AAT CAG ACA AAA CAG TGT GAA CGT TTC AAG TAT GGT GGA TGC	349
Tyr Asn Asn Gln Thr Lys Gln Cys Glu Arg Phe Lys Tyr Gly Gly Cys	
25 30 35	
CTG GGC AAT ATG AAC AAT TTT GAG ACA CTG GAA GAA TGC AAG AAC ATT	397
Leu Gly Asn Met Asn Asn Phe Glu Thr Leu Glu Glu Cys Lys Asn Ile	
40 45 50	
TGT GAA GAT GGT TAATCTAGA	418
Cys Glu Asp Gly	
55	

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 111 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
 -53 -50 -45 -40

Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
 -35 -30 -25

Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Glu
 -20 -15 -10

Arg Leu Glu Lys Arg Lys Pro Asp Phe Cys Phe Leu Glu Glu Asp Pro
 -5 1 5 10

Gly Ile Cys Lys Ala Arg Ile Ile Arg Tyr Phe Tyr Asn Asn Gln Thr
 15 20 25

Lys Gln Cys Glu Arg Phe Lys Tyr Gly Gly Cys Leu Gly Asn Met Asn
 30 35 40

Asn Phe Glu Thr Leu Glu Glu Cys Lys Asn Ile Cys Glu Asp Gly
 45 50 55

(2) INFORMATION FOR SEQ ID NO: 15:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 418 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
 (A) ORGANISM: synthetic

(ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 77..409

(ix) FEATURE:
 (A) NAME/KEY: sig_peptide
 (B) LOCATION: 77..235

(ix) FEATURE:
 (A) NAME/KEY: mat_peptide
 (B) LOCATION: 236..409

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GAATTCATT CAAGAATAGT TCAAACAAGA AGATTACAAA CTATCAATTT CATAACAAT
 ATAAACGACC AAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC
 Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile
 -53 -50 -45

60

109

41

GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu -40 -35 -30	157
ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn -25 -20 -15	205
GTC GCC ATG GCT GAG AGA TTG GAG AAG AGA AAG CCA GAT TTC TGC TTT Val Ala Met Ala Glu Arg Leu Glu Lys Arg Lys Pro Asp Phe Cys Phe -10 -5 1 5	253
TTG GAA GAA GAT CCT GGA ATA TGT AAA GCT CGT ATT ATC AGG TAT TTT Leu Glu Glu Asp Pro Gly Ile Cys Lys Ala Arg Ile Ile Arg Tyr Phe 10 15 20	301
TAT AAC AAT CAG ACA AAA CAG TGT GAA CGT TTC AAG TAT GGT GGA TGC Tyr Asn Asn Gln Thr Lys Gln Cys Glu Arg Phe Lys Tyr Gly Gly Cys 25 30 35	349
AGG GGC AAT ATG AAC AAT TTT GAG ACA CTG GAA GAA TGC AAG AAC ATT Arg Gly Asn Met Asn Asn Phe Glu Thr Leu Glu Glu Cys Lys Asn Ile 40 45 50	397
TGT GAA GAT GGT TAATCTAGA Cys Glu Asp Gly 55	418

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 111 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala -53 -50 -45 -40
Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser -35 -30 -25
Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Glu -20 -15 -10
Arg Leu Glu Lys Arg Lys Pro Asp Phe Cys Phe Leu Glu Glu Asp Pro -5 1 5 10
Gly Ile Cys Lys Ala Arg Ile Ile Arg Tyr Phe Tyr Asn Asn Gln Thr 15 20 25

Lys Gln Cys Glu Arg Phe Lys Tyr Gly Gly Cys Arg Gly Asn Met Asn
 30 35 40

Asn Phe Glu Thr Leu Glu Glu Cys Lys Asn Ile Cys Glu Asp Gly
 45 50 55

(2) INFORMATION FOR SEQ ID NO: 17:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 418 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
 (A) ORGANISM: synthetic

(ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 77..409

(ix) FEATURE:
 (A) NAME/KEY: sig_peptide
 (B) LOCATION: 77..235

(ix) FEATURE:
 (A) NAME/KEY: mat_peptide
 (B) LOCATION: 236..409

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GAATTCATT CAAGAATAGT TCAAACAAGA AGATTACAAA CTATCAATTT CATAACAAT	60
ATAAAGGACC AAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC	109
Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile	
-53 -50 -45	
GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG	157
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu	
-40 -35 -30	
ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC	205
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn	
-25 -20 -15	
GTC GCC ATG GCT GAG AGA TTG GAG AAG AGA AAG CCA GAT TTC TGC TTT	253
Val Ala Met Ala Glu Arg Leu Glu Lys Arg Lys Pro Asp Phe Cys Phe	
-10 -5 1 5	
TTG GAA GAA GAT CCT GGA ATA TGT AAA GCT CGT ATT ATC AGG TAT TTT	301
Leu Glu Glu Asp Pro Gly Ile Cys Lys Ala Arg Ile Ile Arg Tyr Phe	
10 15 20	

43

TAT AAC AAT CAG ACA AAA CAG TGT GAA CGT TTC AAG TAT GGT GGA TGC 349
 Tyr Asn Asn Gln Thr Lys Gln Cys Glu Arg Phe Lys Tyr Gly Gly Cys
 25 30 35
 AGG GGC AAT ATC AAC AAT TTT AAG ACA CTC GAA GAA TGC AAG AAC ATT 397
 Arg Gly Asn Met Asn Asn Phe Lys Thr Leu Glu Glu Cys Lys Asn Ile
 40 45 50
 TGT GAA GAT GGT TAATCTAGA 418
 Cys Glu Asp Gly
 55

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 111 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
 -53 -50 -45 -40
 Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
 -35 -30 -25
 Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Glu
 -20 -15 -10
 Arg Leu Glu Lys Arg Lys Pro Asp Phe Cys Phe Leu Glu Glu Asp Pro
 -5 1 5 10
 Gly Ile Cys Lys Ala Arg Ile Ile Arg Tyr Phe Tyr Asn Asn Gln Thr
 15 20 25
 Lys Gln Cys Glu Arg Phe Lys Tyr Gly Gly Cys Arg Gly Asn Met Asn
 30 35 40
 Asn Phe Lys Thr Leu Glu Glu Cys Lys Asn Ile Cys Glu Asp Gly
 45 50 55

CLAIMS

- 5 1. A variant of human Kunitz-type protease inhibitor domain II of tissue factor pathway inhibitor (TFPI), the variant comprising the following amino acid sequence

10 X¹ Asp Phe Cys Phe Leu Glu Glu Asp X² Gly X³ Cys X⁴ X⁵ X⁶ X⁷ X⁸ X⁹
Tyr Phe Tyr Asn Asn Gln Thr Lys Gln Cys Glu Arg Phe X¹⁰ Tyr Gly
Gly Cys X¹¹ X¹² X¹³ Met Asn Asn Phe X¹⁴ Thr Leu Glu Glu Cys Lys Asn
Ile Cys Glu Asp X¹⁵ (SEQ ID No. 1)

- 15 wherein X¹ represents H or 1-5 naturally occurring amino acid residues except Cys, X²-X¹⁴ each independently represents a naturally occurring amino acid residue, and X¹⁵ represents OH or 1-5 naturally occurring amino acid residues except Cys, with the proviso that at least one of the amino acid residues X¹-X¹⁵ is different from the corresponding amino acid residue of the
20 native sequence.

2. A variant according to claim 1, wherein X¹ is Lys-Pro.

- 25 3. A variant according to claim 1, wherein X² is an amino acid residue selected from the group consisting of Ala, Arg, Thr, Asp, Pro, Glu, Lys, Gln, Ser, Ile and Val.

4. A variant according to claim 3, wherein X² is Thr or Pro.

- 30 5. A variant according to claim 1, wherein X³ is an amino acid residue selected from the group consisting of Pro, Thr, Leu, Arg, Val and Ile.

6. A variant according to claim 5, wherein X³ is Pro or Ile.

- 35 7. A variant according to claim 1, wherein X⁴ is an amino acid residue selected from the group consisting of Lys, Arg, Val, Thr, Ile, Leu, Phe, Gly, Ser, Met, Trp, Tyr, Gln, Asn and Ala.

8. A variant according to claim 7, wherein X⁴ is Lys, Val, Leu, Ile, Thr, Met, Gln or Arg.
9. A variant according to claim 1, wherein X³ is an amino acid residue selected from the group consisting of Ala, Gly, Thr, Arg, Phe, Gln and Asp.
10. A variant according to claim 9, wherein X⁵ is Ala, Thr, Asp or Gly.
11. A variant according to claim 1, wherein X⁶ is an amino acid residue selected from the group consisting of Arg, Ala, Lys, Leu, Gly, His, Ser, Asp, Gln, Glu, Val, Thr, Tyr, Phe, Asn, Ile and Met.
12. A variant according to claim 11, wherein X⁶ is Arg, Phe, Ala, Leu or Tyr.
13. A variant according to claim 1, wherein X⁷ is an amino acid residue selected from the group consisting of Ile, Met, Gln, Glu, Thr, Leu, Val and Phe.
14. A variant according to claim 13, wherein X⁷ is Ile.
15. A variant according to claim 1, wherein X⁸ is an amino acid residue selected from the group consisting of Ile, Thr, Leu, Asn, Lys, Ser, Gln, Glu, Arg, Pro and Phe.
16. A variant according to claim 15, wherein X⁸ is Ile or Thr.
17. A variant according to claim 1, wherein X⁹ is an amino acid residue selected from the group consisting of Arg, Ser, Ala, Gln, Lys and Leu.
18. A variant according to claim 17, wherein X⁹ is Arg.
19. A variant according to claim 1, wherein X¹⁰ is an amino acid residue selected from the group consisting of Gln, Pro, Phe, Ile

Lys, Trp, Ala, Thr, Leu, Ser, Tyr, His, Asp, Met, Arg and Val.

20. A variant according to claim 19, wherein X¹⁰ is Val or Lys.

5 21. A variant according to claim 1, wherein X¹¹ is an amino acid residue selected from the group consisting of Gly, Met, Gln, Glu, Leu, Arg, Lys, Pro and Asn.

22. A variant according to claim 21, wherein X¹¹ is Arg or Leu.

10

23. A variant according to claim 1, wherein X¹² is Ala or Gly.

24. A variant according to claim 1, wherein X¹³ is an amino acid residue selected from the group consisting of Lys, Asn and Asp.

15

25. A variant according to claim 24, wherein X¹³ is Lys or Asn.

20 26. A variant according to claim 1, wherein X¹⁴ is an amino acid residue selected from the group consisting of Val, Tyr, Asp, Glu, Thr, Gly, Leu, Ser, Ile, Gln, His, Asn, Pro, Phe, Met, Ala, Arg, Trp and Lys.

27. A variant according to claim 26, wherein X¹⁴ is Lys or Glu.

25 28. A variant according to claim 1, wherein X¹⁵ is Gly.

29. A variant according to claim 1, wherein X¹ is Lys-Pro and X¹⁵ is Gly.

30 30. A variant according to claim 1 which comprises the following amino acid sequence

35 X¹ Asp Phe Cys Phe Leu Glu Glu Asp X² Gly X³ Cys X⁴ X⁵ X⁶ X⁷ X⁸ X⁹
Tyr Phe Tyr Asn X¹⁶ Gln X¹⁷ Lys Gln Cys Glu Arg Phe X¹⁰ Tyr Gly Gly
Cys X¹¹ X¹² X¹³ Met Asn Asn Phe X¹⁴ Thr Leu Glu Glu Cys Lys Asn Ile
Cys Glu Asp X¹⁵ (SEQ ID No. 2)

wherein X¹-X¹⁵ are as indicated in claim 1, X¹⁶ is an amino acid

residue selected from the group consisting of Gln, Gly, Ala, Ser, Val and Phe, in particular Gln or Ala, and X¹⁷ is an amino acid residue selected from the group consisting of Thr or Ala.

- 5 31. A variant according to claim 1, which has the following amino acid sequence

Lys Pro Asp Phe Cys Phe Leu Glu Glu Asp Pro Gly Ile Cys Lys Ala
Arg Ile Thr Arg Tyr Phe Tyr Asn Asn Gln Thr Lys Gln Cys Glu Arg
10 Phe Lys Tyr Gly Gly Cys Leu Gly Asn Met Asn Asn Phe Glu Thr Leu
Glu Glu Cys Lys Asn Ile Cys Glu Asp Gly (SEQ ID No. 3).

32. A variant according to claim 1, which has the following amino acid sequence

15 Lys Pro Asp Phe Cys Phe Leu Glu Glu Asp Pro Gly Ile Cys Lys Ala
Arg Ile Thr Arg Tyr Phe Tyr Asn Asn Gln Thr Lys Gln Cys Glu Arg
Phe Lys Tyr Gly Gly Cys Arg Gly Asn Met Asn Asn Phe Glu Thr Leu
Glu Glu Cys Lys Asn Ile Cys Glu Asp Gly (SEQ ID No. 4).

- 20 33. A variant according to claim 1, which has the following amino acid sequence

Lys Pro Asp Phe Cys Phe Leu Glu Glu Asp Pro Gly Ile Cys Lys Ala
25 Arg Ile Thr Arg Tyr Phe Tyr Asn Asn Gln Thr Lys Gln Cys Glu Arg
Phe Lys Tyr Gly Gly Cys Arg Gly Asn Met Asn Asn Phe Lys Thr Leu
Glu Glu Cys Lys Asn Ile Cys Glu Asp Gly (SEQ ID No. 5).

- 30 34. A variant according to claim 1 comprising the following amino acid sequence

Lys Pro Asp Phe Cys Phe Leu Glu Glu Asp Pro Gly Pro Cys Lys Ala
Arg Ile Ile Arg Tyr Phe Tyr Asn Asn Gln Thr Lys Gln Cys Glu Arg
Phe Val Tyr Gly Gly Cys Arg Ala Lys Met Asn Asn Phe Lys Thr Leu
35 Glu Glu Cys Lys Asn Ile Cys Glu Asp Gly (SEQ ID No. 6).

35. A DNA construct comprising a DNA sequence encoding a human Kunitz-type protease inhibitor variant according to any of

claims 1-34.

36. A recombinant expression vector comprising a DNA construct according to claim 35.

5

37. A cell containing a DNA construct according to claim 35 or an expression vector according to claim 36.

10 38. A method of producing a human Kunitz-type protease inhibitor variant according to any of claims 1-34, the method comprising culturing a cell according to claim 37 under conditions conducive to the expression of the protein, and recovering the resulting protein from the culture.

15 39. A pharmaceutical composition comprising a human Kunitz-type protease inhibitor variant according to any of claims 1-34 and a pharmaceutically acceptable carrier or excipient.

20 40. A composition according to claim 39 which further comprises heparin.

25 41. Use of human Kunitz-type protease inhibitor domain II of TFPI or a variant thereof according to any of claims 1-34 for the preparation of a medicament for the prevention or treatment of diseases or conditions associated with pathological proteolysis.

1/3

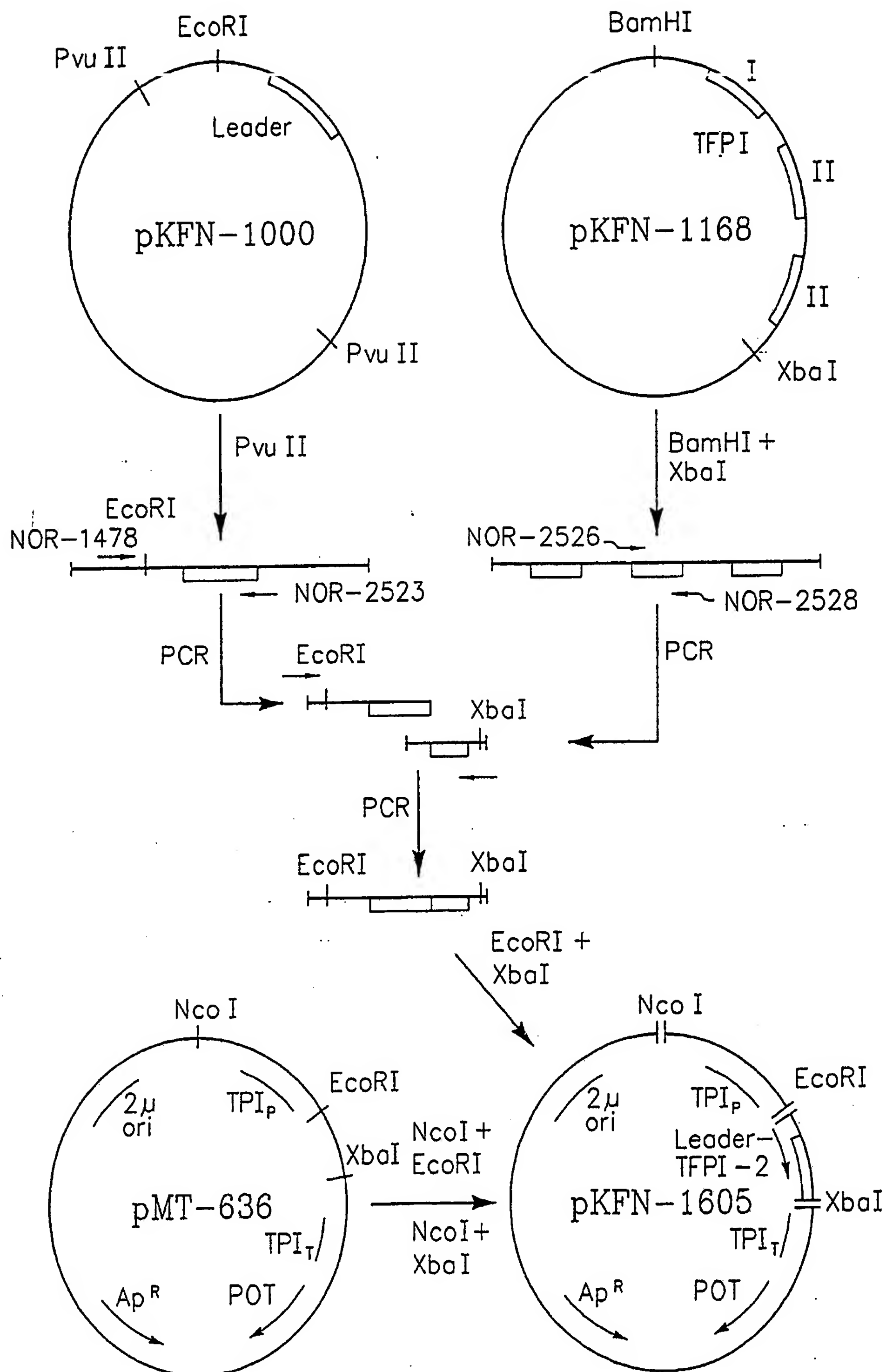


Fig. 1

2/3

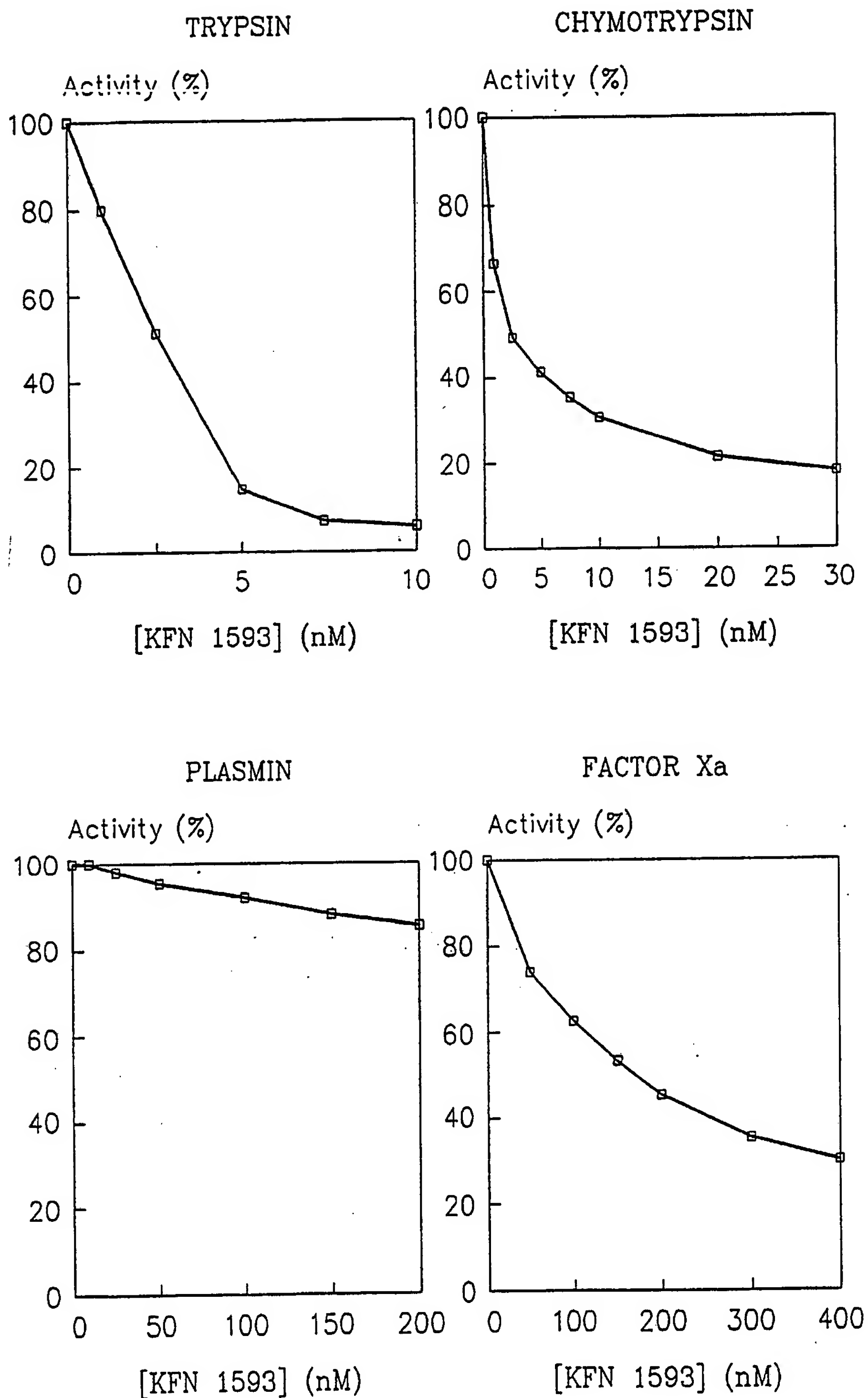


Fig. 2

3/3

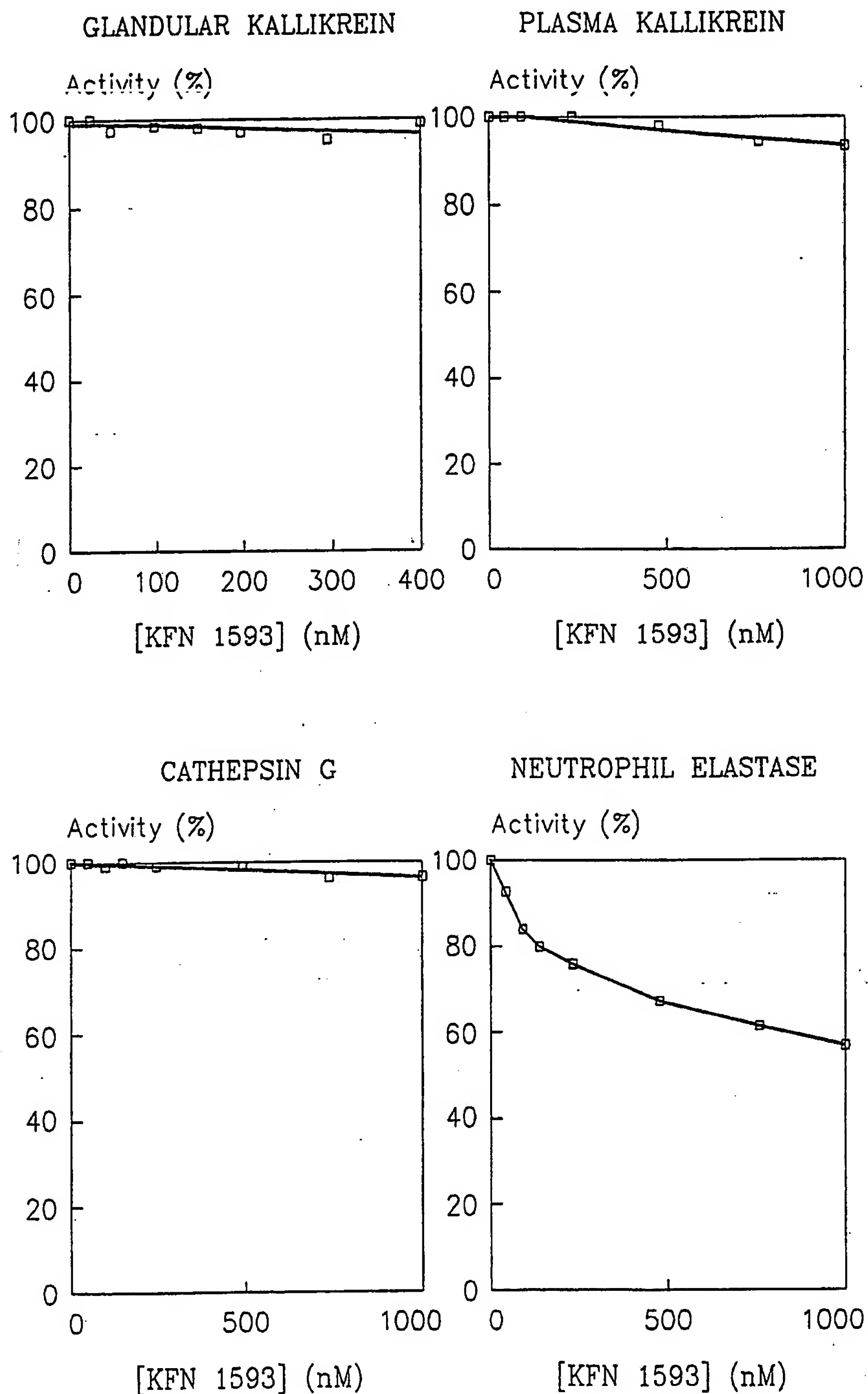


Fig. 3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 93/00004

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: C07K 7/10, C12N 15/15, A61K 37/64

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: A61K, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CHEMICAL ABSTRACTS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NATURE, Volume 338, April 1989, Thomas J. Girard et al, "Functional significance of the Kunitz-type inhibitory domains of lipoprotein-associated coagulation inhibitor" page 518 - page 520	1,7,8,39,41
A	---	2-6,9-38
A,P	US, A, 5106833 (BROZE, JR. ET AL), 21 April 1992 (21.04.92)	1-41

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

7 April 1993

Date of mailing of the international search report

14-04-1993

Name and mailing address of the ISA/

Swedish Patent Office

Box 5055, S-102 42 STOCKHOLM

Facsimile No. +46 8 666 02 86

Authorized officer

Elisabeth Carlborg

Telephone No. +46 8 782 25 00

26/02/93

PCT/DK 93/00004

Form PCT/ISA/210 (parent family annex) (July 1992)